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EDEMA OF THE CORNEAL EPITHELIUM CAUSED BY ATABRINE

OBSERVATION ON THREE PATIENTS

MAJOR FREDERIC M. REESE, MC, AUS
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This report is submitted because it is believed by the author that edema of the corneal epithelium observed in three patients was definitely caused by atabrine. These patients were all admitted to the hospital complaining of severe blurring of vision in both eyes. All had received atabrine for at least several weeks. All developed blurring of vision while taking more than the usual suppressive dose of atabrine. Examination showed generalized edema (bedewing) of the corneal epithelium in both eyes in all three. This disappeared when atabrine was discontinued. These patients were all under treatment in the hospital at the same time (July, 1945). Since they were all assigned to units in malarious areas, where suppressive atabrine was required, and since they might also have to receive therapeutic doses of atabrine at some future date, it seemed to be of vital importance to determine whether the corneal edema had been caused by the atabrine. Each of the patients was therefore given a therapeutic course of atabrine of about half the usual dosage and the condition of the eyes was closely followed by frequent slitlamp examinations. In each instance, after a latent period of several days the corneal edema recurred. When the atabrine was discontinued the corneae again became quite normal.

Case 1. Staff Sergeant Age 24. This soldier was admitted to the hospital complaining of severe blurring of vision for six days. He was led into the clinic by another soldier and obviously was unable to see well enough to get about in bright daylight. His general health had been excellent except for a malarial chill 21 days previously. Following this chill he had received 1.2 grams of atabrine a day for six days and then 0.2 gram a day until the day of admission here. He had experienced no discomfort except for slight "scratchiness" in both eyes just before blurring of vision began.

Examination showed both eyes to be entirely free of inflammation and there was no tearing or photophobia. There was however, a uniform haziness of both corneae of severe degree. Examination with the slitlamp which caused no more discomfort than would be expected if his eyes had been normal, showed a uniform "bedewing" of the epithelium of both corneae of unusually fine texture extending from limbus to limbus. No bullae were observed and there was no staining with flourescein solution. No dilatation of the pericorneal vessels could be seen. No details of the deeper tissues or fluid spaces beneath the corneae could be made out except that it could be ascertained that the anterior chambers were of approximately normal depth and that the pupils were of average size, and reactive. It was not thought wise to place a heavy McLean tonometer on the corneae, however, the intraocular tension was normal to palpation. The fundi, of course, could not be seen. Visual acuity was reduced to "hand movements" in the right eye and ability to count fingers in the left eye.

A general physical examination showed no abnormality. The patient was well developed, well nourished, mentally alert and quite cooperative. Laboratory studies of the blood were negative. The urine, however, showed one to eight RBC per HPF. This cleared entirely after fourteen days.

On the date of admission to the hospital the significance of atabrine in regard to the patients' condition was not appreciated. He therefore received the usual suppressive dose of 0.1 gram a day for the next four weeks. Local treatment consisting of boric ointment followed by hot compresses for fifteen minutes every two hours during the day was started. For three days the eyes were covered by eye pads. By this time definite clearing of the corneae was evident and the patient was able to get about during the day. Eleven days after admission his visual acuity had improved to 20/30 in each eye and changes in the corneal epithelium could be seen only with the slitlamp. With this degree of improvement examination further showed no abnormal changes in the deeper layers of the corneae. The aqueous ray was negative and the iris and lens of each eye were normal. No vitreous opacities were noted and the fundi were entirely normal. The visual fields were normal. During this four weeks period, during which the patient received 0.1 gram of atabrine a day, examination

without magnification showed no abnormality of either eye, visual acuity was normal and the patient was entirely comfortable. Examination with the slitlamp however, showed scattered isolated "points" of edema in the corneal epithelium of both eyes. At the end of this four weeks' period the daily atabrine tablet was discontinued and 0.6 gram of quinine was substituted. Three weeks later these isolated "points" of edema had completely disappeared and the corneae appeared normal. Seven weeks after admission to the hospital slitlamp examination showed no abnormality of either cornea for the first time. The following course of atabrine was started: 0.6 gram of atabrine a day for three days, 0.2 gram for three days and 0.1 gram a day until such time as corneal changes might appear. Four days after this course of treatment was started, changes apparent only by slitlamp examinations appeared and then increased steadily for the next ten days when the corneae showed severe "bedewing" of the epithelium and visual acuity was again reduced to "hand movements" in each eye. Again no discomfort was experienced by the patient except for a sensation of "slight roughness" in both eyes on the twelfth day after the atabrine was started. Maximum changes developed, therefore, in both corneae fourteen days after the atabrine was started. The drug was discontinued at this time and 0.6 gram of quinine a day substituted. No improvement whatever could be made out for the next six days. Ten days after the atabrine was stopped, however, the patient stated that he could see better, but little change in the corneal epithelium could be made out with the slitlamp. Fourteen days after the atabrine was stopped visual acuity had improved to 20/40 in each eye and the corneae were obviously clearing. Twenty-four days after the atabrine was stopped the corneae appeared normal by slitlamp examination and visual acuity was 20/30 in each eye and 20/20-2 with both eyes. The patient was entirely comfortable.

Case II. Pfc. Age 31. This soldier was also admitted to the hospital complaining of severe blurring of vision for five days. His health had been excellent except for fifteen attacks of malaria over a period of almost two years. His last chill had occurred approximately two months previously. For the last six or eight months he had been aware of transient attacks of blurring of vision lasting from thirty minutes to four hours. These attacks were never severe and usually occurred

when he was hot and perspiring freely. He had never associated them with his antimalaria therapy. Following each chill he had received 0.6 gram of atabrine and 1.8 grams of quinine a day for three days and then 0.2 gram of atabrine a day for two days followed by the regular suppressive dose of 0.1 gram a day. During the week before admission to this hospital, however, he had received 0.2 gram of atabrine a day.

Examination of this soldier's eyes on the day of admission to this hospital showed precisely the same changes in the corneal epithelium of both eyes described in case 1 except they were not so intense. Visual acuity was 20/2001 in the right eye and 18/200 in the left eye. The intraocular tension was normal to palpation and the rate of tear flow was normal as measured by the Schirmer test. The pupils were dilated, however, as a result of a previously instilled cycloplegia.

General physical examination showed no significant abnormalities. The patient was robust, well nourished, mentally alert and cooperative. He had no complaints except for the loss of vision. Laboratory studies were negative except for an eosinophilia in the blood of 17% which could not be accounted for by repeated stool examinations.

This patient was also put on 0.1 gram of atabrine a day. Local treatment consisting of boric ointment and hot compresses for fifteen minutes every two hours during the day was started. Although the suppressive dose (0.1 gram a day) was continued for the next seventeen days, edema of the corneal epithelium diminished rapidly and visual acuity improved to 20/20 in each eye within five days. However, scattered "points" of edema in the corneal epithelium could be seen with the slitlamp as long as this dose of atabrine was continued. When 0.1 gram a day of atabrine was stopped seventeen days after admission to the hospital 0.6 gram of quinine was substituted. Thirty eight days after admission to the hospital and twenty-one days after stopping atabrine, visual acuity was 20/20 in each eye and slitlamp examination showed that both corneae were normal. As in case 1 the following course of atabrine was started: 0.6 gram of atabrine a day for three days, 0.2 gram of atabrine a day for three days and 0.1 gram a day until such time as corneal changes might appear. Six days after this therapeutic course was started, no changes in the corneal epithelium of either eye could be made out with the slitlamp. Nine days after it was started, extensive and widespread edema "bedewing"

of the corneal epithelium of both eyes was apparent with or without the slitlamp. Visual acuity was reduced to approximately 16/200 in each eye. Both sclerae remained entirely white, however, and free of inflammation and there was no dilatation of the pericorneal vessels in either eye. The patient complained of no discomfort except for the visual loss. Nine days after the atabrine had been started it was discontinued and 0.6 gram of quinine a day was substituted. No improvement whatever was noted in either eye for the following thirteen days. During the next four days, however, the patient noted gradual and definite improvement in his vision and seventeen days after the atabrine was stopped visual acuity had improved to 20/20 in each eye. Isolated "points" of edema in the corneal epithelium of both eyes, however, could be seen with the slitlamp. Twenty-five days after the atabrine was stopped both corneae were entirely normal by slitlamp examination and visual acuity was 20/20 in each eye.

Case III. Pvt. Age 21. This soldier was admitted to the hospital complaining of severe blurring of vision for twenty-eight days. Atabrine had been taken for a total of sixty-three days, 0.1 gram a day for the first twenty-seven days, 0.2 gram a day for the next twenty-seven days and 0.1 gram a day for the last nine days before admission to the hospital. Blurring of vision began eight days after the dose of atabrine had been increased to 0.2 gram a day. It remained severe until three days after the dose had again been decreased to 0.1 gram a day. Improvement had apparently been rapid, for on the day of admission, nine days after the dose had been decreased to 0.1 gram a day, only isolated "points" of edema in the corneal epithelium could be made out with the slitlamp and visual acuity was 20/30 in the right eye and 20/20 in the left eye. Examination showed no other abnormal changes. The intraocular tension was normal to palpation and the rate of tear flow was normal by the Schirmer test.

General physical examination disclosed no significant abnormalities except for the eyes. The patient was well devoped, well nourished, mentally alert and quite cooperative. He had no complaints except for loss of vision. Laboratory studies of the blood and urine were negative.

Atabrine was discontinued the day after the patient was admitted to the hospital. The "points" of corneal epithelial edema, apparent

only by slitlamp examination, persisted for two weeks. During this time the patient was entirely comfortable and his visual acuity was normal. After all evidences of epithelial edema by slitlamp examination had disappeared, the following course of atabrine was given: 0.6 gram a day for three days, 0.2 gram a day for three days and then 0.1 gram a day until changes in the corneal epithelium developed. Three days after this course of atabrine was started, isolated "points" of edema in the corneal epithelium of each eye could be seen with the slitlamp. These changes gradually increased in intensity until generalized "bedewing" of the corneal epithelium was apparent nine days after the atabrine was started and visual acuity was reduced to about 10/200 in each eye. In spite of the extensive changes in the corneal epithelium there was no dilatation of the pericorneal vessels. The eyes were quite free of inflammation. The atabrine was discontinued nine days after the experimental therapeutic course had been started. Three days later the patient seemed to be aware of some improvement although examination with the slitlamp showed no change for the better. Twelve days after the atabrine had been discontinued however, visual acuity had improved to 20/30-3 in each eye and marked thinning of the edema in the corneal epithelium was apparent by slitlamp examination. Fourteen days after the drug had been stopped visual acuity had improved to 20/20 in each eye and only very occasional "points" of edema in the corneal epithelium could be seen with the slitlamp. The patient was evacuated to the U. S. A. four days later.

SUMMARY

A summary is presented of the case records of three patients admitted to this hospital complaining of severe blurring of vision in both eyes which developed while each was taking 0.2 gram of atabrine a day. One patient had received a larger therapeutic course of atabrine twenty-one days previously, the second had received therapeutic courses of the drug repeatedly for almost two years up to two months previously and the third patient had received 0.2 gram of atabrine a day for twenty-seven days up to nine days before admission to this hospital. Examinations of these patients' eyes showed edema of the corneal epithelium, "bedewing" of an unusually fine texture

and uniform appearance with the slitlamp. Bullae were never seen and staining with flourescein solution never occurred. Dilatation of the pericorneal vessels or other signs of irritation were never noted. Examination of the eyes otherwise showed no abnormalities. General physical examination of all three disclosed no other significant abnormality. All three soldiers appeared to be in excellent health. There was no dermatitis or other skin eruption. An eosinophilia in one could not be accounted for by repeated stool examinations. In case I and II minimal corneal changes visible only by slitlamp examination persisted for three weeks when 0.1 gram of atabrine a day was taken. These changes finally disappeared three weeks after the drug had been discontinued and 0.6 gram of quinine a day substituted. A small therapeutic course of atabrine given to each patient after all slitlamp signs of epithelial edema had disappeared, resulted in a full-blown recurrence in all three. Discontinuance of atabrine a second time in all three with substitution of 0.6 gram of quinine per day in two, again resulted in a disappearance of the corneal epithelial edema.

CONCLUSIONS

Observations on three patients indicate definitely that edema of the corneal epithelium in all three was related to atabrine. In each instance the visual disturbance was most marked when the patients were receiving more than the usual suppressive dose of atabrine (0.1 gram per day). However, reduction of the dosage to the usual suppressive level did not suffice to clear up the condition entirely. Changes in the corneal epithelium visible by slitlamp examination did not disappear completely until about three weeks after atabrine had been discontinued. Recovery was not appreciably retarded by the administration of a daily suppressive dose of quinine (0.6 gram per day). This process is apparently reversible and does not result in any permanent damage to the ocular tissues.

ADDENDUM

After the completion of this report a fourth patient with edema of the corneal epithelium of both eyes was transferred to this hospital with a diagnosis of: 1. Cyclitis, non suppurative, acute, bilateral, moderately severe, cause undetermined, vision 20/30 in each eye,

and 2. Hepatitis, infectious, acute moderately severe, cause undetermined. He gave a history of having had several elevations of temperature with chills over a period of eight months. A positive smear for malaria, however, was never obtained. Diarrhea had occurred on several occasions but stool examinations were all negative. About fifty-one days before his transfer to this hospital he developed fever, generalized aching and jaundice and a diagnosis of infectious jaundice was made. Malaria, however, was suspected, for, although he had been on a regular suppressive dose of atabrine of 0.1 gram daily, between the forty-fourth and thirty-ninth days before transfer here he received 1.2 grams of atabrine a day and on the forty-second day developed severe blurring of vision in both eyes which persisted for about three weeks and then gradually improved. He had received 0.1 gram of atabrine a day until he arrived here.

Examination on admission showed edema of the corneal epithelium of both eyes of mild degree unassociated with any other ocular changes. Visual acuity was 20/30 in each eye.

General physical examination made by the medical consultant corroborated the diagnosis of infectious jaundice. The icteric index was found to be 37.

Atabrine was stopped immediately and 0.6 gram of quinine a day substituted. Two weeks later examination with the slitlamp showed marked improvement and visual acuity was improved to 20/20 in each eye. The patient was evacuated to the U. S. A.

SODIUM CAPRYLATE

A New and Effective Treatment for Moniliasis of the Skin and Mucous Membranes*

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The Moniliae are yeast-like non ascosporogenous fungi. Of the many species included in the genus Monilia, only one, Monilia albicans, is commonly pathogenic for man. The clinical manifestations of infections produced by this organism vary greatly and are dependent upon the region of the body affected. Moniliasis, therefore, is a term which may designate an acute or chronic infection produced by the Moniliae, occurring in the mouth, vagina, skin, nails, bronchi, lungs and infrequently in the endocardium and meninges.

It is important to call to the attention of the readers that mycologists have replaced the familiar generic name Monilia by the name Candida. The name of the fungus, Monilia albicans, therefore becomes Candida albicans. Many texts on medical mycology in an attempt to be erudite use the name Candida albicans for the fungus, but at the same time retain the term Moniliasis for the disease processes that are produced. This confusion in taxonomy is the despair of the clinician. The terms Monilia albicans and Moniliasis are retained in this paper because they are commonly used in medical writings. Perhaps it is timely to suggest that if physicians adopt the generic name Candida in place of Monilia that the name Moniliasis also be replaced by either "Candidiasis" or "Candidosis".

The current methods of treating Moniliasis of the skin and mucous membranes are not satisfactory. Gentian violet, diluted 1:10,000 is commonly employed in infections of the mouth and vagina and in a 1 per cent solution it is used for cutaneous infections. The results obtained with gentian violet are not impressive enough to counteract the obviously disagreeable features that accompany its use. There is then a distinct need for a therapeutic agent which will eradicate the fungus

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Johns Hopkins University.

and which can be applied to large surfaces of the skin and mucous membranes without producing irritation and without inflicting upon the patient a sensation of embarrassment due to its presence.

The sodium salts of propionic, valeric, caproic, caprylic, capric and undecylenic acids are fungistatic and fungicidal in different degrees for various pathogenic fungi when tested by in vitro methods (1, 2, 3). Of these fatty acid salts sodium caprylate was particularly effective against Monilia albicans (3). The possibility then arose that sodium caprylate incorporated in suitable vehicles might be effective clinically in the treatment of fungous infections due to Monilia albicans. The opportunity to use sodium caprylate in vivo first occurred in patients with thrush. The following case report illustrates its dramatic effects in what had proved to be a prolonged, slowly progressing infection that had been resistant to treatment.

REPORT OF CASE

B. H., a 2 year old white female was first seen on December 3, 1945. Her parents, 6 months previously, had noticed a "peculiar white patch the size of a penny on her palate." This white patch had slowly increased in size despite the treatments of several physicians. The lesion had been diagnosed as thrush and Monilia albicans and had been cultured. Prolonged and frequent local treatment with gentian violet, sulfadiazine and penicillin had been tried. Regardless of this the lesion had continued to spread.

The physical examination, except for the lesion in the mouth, was essentially negative. Over most of the left side of the soft palate and extending to the right side of the soft palate and down over the anterior pillar of the left tonsil was a white, rough, membranous-like patch (Fig. 1). The mucous membrane surrounding the patch was slightly inflamed for a distance of about 2 mm. The lesion was not painful to touch. My first impression was that the patch could be scraped off as if it were a diphtheritic membrane. This was certainly not the case because the lesion was attached firmly to the underlying mucous membrane and it was only by extreme patience on our part that we were able to scrape enough away for examination and culturing.

Mats of mycelia were easily found by examining the scrapings directly under the microscope. Other scrapings were inoculated on Sabouraud's dextrose agar and incubated at 30° centigrade. After 4 days a creamy moist colony appeared and a portion of this was transferred to a corn meal agar plate and other portions were transplanted into sugar, beef extract broth tubes. On corn meal agar the organism rapidly produced mycelium-bearing, ball-like clusters of budding cells and in the sugar tubes the organism produced acid and gas in glucose and maltose and acid alone in sucrose. The cultural characteristics of the organism were typically those of Monilia albicans.

A 20 per cent aqueous solution of sodium caprylate adjusted to pH 7.4 was prepared. The parents were advised to apply this solution locally to the lesion with a large swab every 2 hours during the day. They were also advised to swab the entire mouth with the solution 3 times daily.

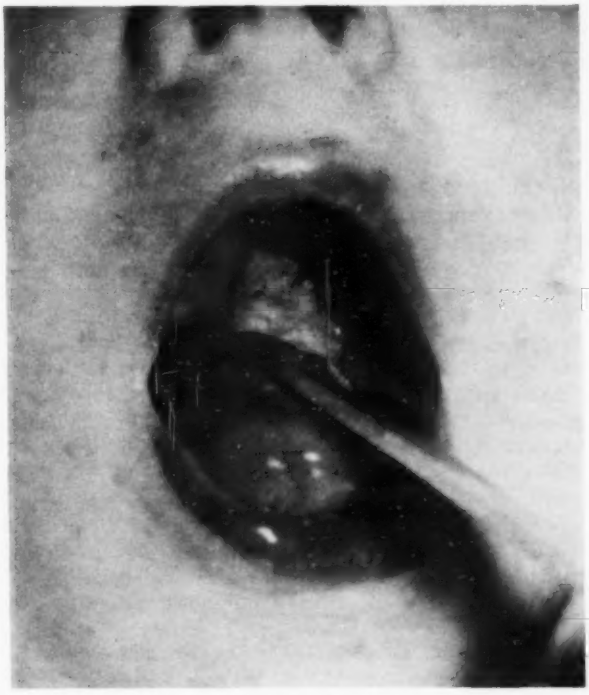

FIG. 1. THRUSH DUE TO MONILIA ALBICANS

Lesion disappeared after 20 days of treatment with 20% sodium caprylate solution

After 20 days of such treatment the lesion had completely disappeared. It has now been 1 month since the treatment was discontinued and there has been no recurrence of the infection.

Equally as dramatic have been the results of treatment with sodium caprylate in 5 additional patients with thrush. The patients were all infants or young children with lesions not quite so extensive as the one just described in the case report. The method of administering treat-

ment was identical to that just described, but the period of treatment necessary to effect a cure was shorter. The lesions disappeared in these patients after 4 to 10 days of treatment.

The results in the treatment of thrush due to Monilia albicans would probably not have been reported at this time had it not been for the unusually dramatic results that were again obtained with sodium caprylate and in this instance in the treatment of an adult patient with extensive cutaneous and mucous membrane Moniliasis.

For the treatment of this patient a 10 per cent sodium caprylate ointment[1] and a 10 per cent sodium caprylate vaginal jelly[2] were prepared. The sodium caprylate ointment has been tested in vitro against Monilia albicans by the agar cup plate technique and the results reported elsewhere (4).

REPORT OF CASE

V. C., a 47 year old white married woman was first seen August 10th, 1945, complaining of "itching and eczema". About the first of January, 1945, she became aware of "cracks" in the corners of her mouth. These lesions were treated with several ointments by her physician without effectiveness. She was then given "vitamin tablets" to take, but the lesions still persisted. In April of 1945 she developed an intense itching inside the vagina and soon thereafter an eruption appeared about the vulva which gradually spread to the skin of the groin. At this time she consulted a gynecologist who treated her with "various douches and ointments". She did not improve. By June 1945 an eruption appeared over both lower arms and gradually spread to the skin over the upper arms. By July 1945 small "blisters" and "bumps" began to appear over both lower arms and the skin in the webs of her hands began to "crack open". Concomitantly a "breaking out" over the neck appeared. The past and family histories were irrelevant.

The physical examination, except for the lesions of the skin and mucous membranes, was essentially negative. The skin of the corners of the mouth was macerated, moist, erythematous and fissured. The buccal mucous membranes appeared normal. There was an eczematous eruption over the skin of the neck, both lower arms and the lower one-third of the upper arms. Superimposed over the eczema

[1] Sodium caprylate ointment. Caprylic acid 10.0; diethylene glycol monoethyl ether 3.0; "carbowax (6000)" 47.5; n-propyl alcohol 10.0; zinc caprylate 5.0; sodium hydroxide 2.45; water 22.05. Enough sodium hydroxide is added to adjust to pH 8.0.

[2] Sodium caprylate vaginal jelly. Caprylic acid 10.0; glycerine 10.0; gum tragacanth 12.0; gum acacia 9.0; water approximately 55.0. Enough sodium hydroxide is added to adjust to pH 6.75. The ointment and the jelly were prepared for us by Mycoloid Laboratories, Inc., Little Falls, New Jersey.

of the lower arms were numerous vesicles and pustules. On the skin of the approximating surfaces of the second and third fingers of the right hand and the third and fourth fingers of the left hand were well defined, erythematous, exudating patches of dermatitis which were rimmed by small vesicles. The skin of the webs of these fingers was cracked, moist, red and macerated. The finger nails and skin about the nails were normal. The vulva was red and swollen. There was an eczematoid dermatitis which extended from the vulva to the skin of the groin and down the inner surfaces of both thighs. This dermatitis extended also around the anus and to both cheeks of the buttocks. The mucous membrane of the vagina was fiery red and there were many small white patches scattered over the mucosa. There were no lesions on the skin and nails of the feet.

Laboratory examinations revealed a normal blood morphology, a negative blood Wassermann reaction and a normal urinalysis. Scrapings from the lesions of the corners of the mouth, the skin of the neck, the pustules on the lower arms, the affected webs of both hands, the groin and the vagina were cultured on Sabouraud's dextrose agar and incubated at 30°C. After 5 days a creamy, moist colony began to appear on all cultures except the ones taken from the neck. The odor of the cultures was yeasty. The organisms were picked from the Sabouraud's dextrose agar slants and inoculated on corn meal agar plates by making deep cuts in the agar. At the same time 4 beef extract broth tubes containing 1 per cent glucose, sucrose, lactose and maltose, respectively, were inoculated from each culture. On the corn meal agar plates the organisms rapidly produced mycelium-bearing, ball-like clusters of budding cells and thick-walled, round chlamydospores. The organism produced acid and gas in glucose and maltose and acid in sucrose. The cultural characteristics of the organism were typically those of Monilia albicans.

The patient was instructed to apply sodium caprylate ointment to the corners of the mouth, over the arms and affected parts of the hands and over the skin of the vulva, thigh and buttocks twice daily. She was also instructed to instill into the vagina, with an applicator, sodium caprylate jelly twice daily. The eczematous skin of the neck was not treated. Since the culture from this area had been negative for Monilia albicans, it was believed that the lesion was a hypersensitive manifestation of the infection.

The patient was seen again September 1, 1945. She had then treated herself for 2 weeks. There was a dramatic improvement. The skin of the corners of the mouth was pink, dry and scaling. There were no fissures. The skin of the arms was essentially normal except for a few scattered papules. The lesions between the fingers were healing and the fissures in the webs on both hands had disappeared. There was still an eczematoid dermatitis of the vulva, thigh and buttocks, but the area of skin involvement was less and one was given the impression that the lesion was healing. The itching had nearly subsided in the vagina. The mucous membrane of the vagina did not appear so inflamed as it had on the original examination and the white patches had completely disappeared. The patient complained that the vaginal jelly burned for about 30 minutes after its instillation. The ointment, however, did not burn or irritate the skin. She was instructed to continue with the same routine of treatment.

The patient was seen again September 9, 1945. She was now well except for a small area of eczematoid dermatitis over the skin of the thigh. The itching in the vagina had completely subsided but she still complained of the intense burning from the application of the vaginal jelly. Scrapings from the corners of the mouth, the webs of the hands, the vagina and the groin were taken and cultured on Sabouraud's dextrose agar. There was no growth in any of the tubes. The patient was instructed to continue the use of the ointment in the corners of the mouth and groin but treatment of the vagina was discontinued.

By October 3, 1945 the dermatitis in the groin had about disappeared and itching in the vagina had recurred. Cultures from the vagina and groin were sterile. On the advice of Dr. Houston Everett a 10 per cent sodium caprylate talcum powder mixture was prepared and this powder was blown into the vagina 3 times weekly. After 3 treatments the patient's symptoms disappeared and have not recurred to date. Vaginal burning occurred after the insufflation of the powder but the irritation was less obnoxious to the patient than that produced by the jelly.

SUMMARY AND CONCLUSIONS

The sodium salt of caprylic acid has been introduced for the treatment of mucous membrane and cutaneous infections due to Monilia albicans.

A 20 per cent aqueous solution of sodium caprylate at pH 7.4, administered locally, has been effective in the treatment of 6 patients with thrush due to Monilia albicans.

A 10 per cent sodium caprylate ointment was used successfully in the treatment of a patient with extensive cutaneous Moniliasis. The response to the therapy was prompt and dramatic and there was no evidence of irritation.

A 10 per cent sodium caprylate jelly and powder were employed in treating the same patient for Monilia vaginitis. The results were highly successful but both preparations were irritating. The vaginal insufflation of powder caused less discomfort than the use of the vaginal jelly.

It will occur to the readers of this report that sodium caprylate might be administered parenterally in the treatment of systemic infections produced by Monilia albicans. The author has administered sodium caprylate intravenously and its protracted use is made impossible because of its sclerosing action on veins.

The genus name, Monilia, has been retained in this paper because the term is so ingrained in medical writings. However, it has been sug-

gested that if physicians accept Candida for the generic name of the fungus, that "Candidiasis" or "Candidosis" be adopted to replace Moniliasis as a term connoting the disease processes that this yeast-like fungus produces.

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TOXIC AND IMMUNOLOGICAL PROPERTIES OF PNEUMOCOCCAL HEMOLYSIN[1]

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The hemolytic substance produced by pneumococci is also of toxic nature, as was first clearly indicated by Cole (4). His conclusion that this antigenic hemolysin is a species-specific cellular constituent was substantiated by Neill *et al.* (13, 14). Although Cole noted a general, but not exact, correlation between the lethal toxicity for guinea pigs and the hemolytic activity of his preparations, Neill *et al.* (15) refrained from assigning a lethal toxicity to the hemolysin because of the presence of other toxic endocellular substances in the crude extracts employed.

At different times, various extracts, filtrates, and autolysates of the pneumococcus have been described (22) as having one or another form of toxicity in normal or sensitized animals; but these are of little or no direct concern here, except perhaps the anaerobic autolysate described by Parker (16). An extract of this autolyzed cellular material was reported to possess lung-toxic, dermonecrotic, and hemolytic activities that were species-specific and sensitive to heat and oxidation. Parker regarded the three activities of such autolysates as due to separate entities.

The method devised by Cohen, Halbert and Perkins (2) for the preparation of a concentrate of the pneumococcal hemolysin has made available material of relatively high hemolytic potency. The concentrate was so prepared as to retain practically all of the hemolytic activity that had developed in the cells during their culture and subsequent manipulation. The preparations employed in the following series of experiments were all derived from an avirulent culture (D/39/R) of Type II pneumococci and contained no demonstrable capsular polysaccharide. Analyses showed them to be protein in nature, with 13 to 15% nitrogen and 1 to 2% phosphorus. For the present, the hemolytic potency of a preparation must serve as the

[1] The subject matter of this paper was presented at the Baltimore meeting of the Society of American Bacteriologists, Dec. 31, 1941 (8). Preparation for publication was delayed by the war emergency.

criterion of its relative purity. The most active preparation contained 6000 hemolytic units (HU) per mg. (1 HU is represented by the 50% hemolysis of 4 ml. of a 1% suspension of rabbit erythrocytes in phosphate buffer, pH 7.6). It will be recalled that the hemolytic activity of the native lysin is reversibly abolished when the substance is reversibly oxidized; while over-oxidation results in irreversible loss of the activity (13, 18).

The preparations were employed in a study of the following biological and immunological properties of the hemolysin, namely: lethal toxicity, dermotoxic activity, antigenicity, and possible relation to the C carbohydrate. In most cases two or more of the preparations were employed to check or extend the observations.

EXPERIMENTAL

Lethal Toxicity

The observations were made mostly on mice injected intravenously with buffered solutions of the hemolysin preparations. Occasional experiments indicated that a lethal dose administered intraperitoneally was several times greater than the intravenous; and death occurred more slowly after an intraperitoneal injection. Similar doses administered intradermally did not cause death.

Most of the mice receiving a fatal intravenous dose of the hemolysin died within 2 to 10 minutes, a few within several hours, and an occasional one in one day. Death was preceded by generalized convulsions, gasping respiration, and often by a bloody, frothy, nasal discharge. Purpuric manifestations were never observed. In animals that had died quickly, the only gross changes seen at autopsy were mild to marked pulmonary congestion. Occasionally, in animals taking longer to die, a clear pleural effusion was present; hemoglobinuria was never observed.

Microscopically,[2] evidences only of mild pulmonary congestion to frank pulmonary edema were regularly noted in the animals that died after injection of the hemolysin. No definite lesions were seen in the heart, liver or kidney, except for renal casts and subcutaneous edema in two out of 30 mice. None of the tissues examined showed evidence

[2] We are indebted to Dr. Arnold R. Rich and Dr. Wm. L. Straus, Jr. for aid in interpreting the tissue sections.

of gross intravascular hemolysis. This is in agreement with the observations of Cole (4). This fact does not, however, exclude the possible occurrence of a small degree of hemolysis in the few animals that lingered as long as one day, or perhaps even in the majority that died more quickly. The tissues of surviving mice were not examined.

The heart blood of mice that died rapidly showed no gross signs of hemolysis. Even incubation of the blood for 1 to 2 hours at 37°C failed to result in noticeable hemolytic changes. It was observed incidentally that the washed erythrocytes of the CFl mouse were much more resistant to the hemolysin than were those of the rabbit, guinea pig, or man, and the kinetics of hemolysis of the CFl erythrocytes was abnormal.

Toxicity of the active hemolysin. Pure-strain (CFl) white mice 3 to 4 months old and weighing 20 to 25 gm. were used as the test animals, and the maximum volume injected was 0.2 ml. The animals were injected intravenously with graded amounts of concentrate 60 IX dissolved in phosphate buffer, pH 7.6. This was a preparation of average potency and contained, per mg., 1250 HU of active hemolysin and 150 HU of reversibly oxidized (hemolytically inactive) hemolysin. A toxicity curve obtained by the method of Reed and Muench (17) yielded a value of 0.12 ± 0.01 mg. for the LD_{50}, as shown by the data in Table I. This value is probably 10 per cent too high, because preparation 60 IX was known to contain that proportion of reversibly oxidized hemolysin which is relatively non-toxic (see later). In a preliminary test, another preparation, 59A, with nearly the hemolytic activity of 60 IX, yielded an LD_{50} of 0.13 mg.

Subsequently, the more potent and fully active preparation 63A2 became available for examination. This contained 6000 HU per mg., all of which was hemolytically active; and it was found, in a preliminary test, to yield an LD_{50} of close to 0.04 mg. Of interest in this connection is the report (10) that purified streptolysin O killed 2 out of 4 mice in doses of 0.044 to 0.048 mg., roughly the same order of lethal toxicity as that of our most active pneumococcal hemolysin, 63A2.

The fact that the LD_{50} is 0.12 to 0.13 mg. for concentrates of 1400 to 1500 HU per mg. and 0.04 mg. for a concentrate of 6000 HU per mg. indicates the general correlation of the acute lethal toxicity with the hemolytic activity of the hemolysin. However, a comparison of the

hemolytic units per toxic dose of preparation 63A2 (240 HU) and of 60 IX (170 HU) suggests that the preparation of lower toxicity probably contained non-hemolytic substances of low-grade toxicity which are commonly found in crude extracts of pneumococci (15).

Toxicity of the reversibly oxidized hemolysin. For testing the effect of reversible oxidation of the hemolysin upon its lethal activity, there was available preparation 61-2CCC that had been 90 per cent oxidized. In this state, it possessed a residual hemolytic activity of 180 HU per mg.; and when it was activated with cysteine the hemolytic activity rose to 1800 HU per mg.

A solution of 1 mg. per ml. was made in buffer pH 7.6, and one-half of it was reduced with an excess of cysteine (0.2 ml. of 0.06 M cysteine per ml. of hemolysin solution). Three mice were then injected intravenously with 0.2 mg. of the untreated, oxidized preparation (36 active HU, 360 active plus potential HU); 4 mice were similarly injected with 0.2 mg. of the cysteine-treated preparation (360 active HU); and 3 mice received the amount of cysteine used for reactivation. A sample of the results follows:

TABLE I

Determination of the LD_{50} of concentrate 60 IX in mice

DOSE	ALIVE	DEAD	CUMULATIVE TOTALS		CUMULATIVE PER CENT MORTALITY
			Alive	Dead	
mg.					
0.05	6	0	19	0	0
.075	4	2	13	2	13
.10	3	3	9	5	36
.125	3	3	6	8	57
.15	2	4	3	12	80
.175	1	5	1	17	94
.20	0	6	0	23	100

LD_{50} by interpolation, 0.12 mg. (170 HU).
Standard error = 0.01 mg.

90% Reversibly oxidized hemolysin:	S,	S,	S		
Same, reduced with cysteine:	D2′,	D4′,	D5′,	D40′	
Cysteine controls:	S,	S,	S		

S = survived 4 days and discarded.
D2′ = died in 2 minutes, etc., after injection.

This experiment shows that the 90% reversibly oxidized hemolysin was non-toxic in the dose administered, whereas the reduced hemolysin was toxic. (Herbert and Todd (10) found that streptolysin O had a much greater lethal effect when activated with cysteine). Tests of the toxicity of higher doses of the reversibly oxidized hemolysin must await the production of fully oxidized preparations.

Toxicity of the heat-inactivated hemolysin. Active preparation 59–61 (2400 HU per mg.) was dissolved in the proportion of 1 mg. per ml. of buffer pH 7.6, and the solution divided into two parts. One part was heated at 60° for 10 minutes and then chilled; it had no hemolytic activity. The other part was untreated. Four mice were injected intravenously with 0.25 mg. of the heated hemolysin, and four mice with 0.25 mg. (600 HU, well above the L_{100} dose) of the unheated hemolysin. The results were:

Unheated hemolysin:	D1′,	D1.5′	D2′,	D2′
Heated hemolysin:	S,	S,	S,	S

It is evident therefore that the toxic as well as the hemolytic activity of the concentrate was abolished by heating at 60° for 10 minutes.

Toxicity of the cholesterol-treated hemolysin. A solution of concentrate 60 IX (2 mg. per ml. of buffer pH 7.6) was divided into two parts. The first, not further treated, was injected intravenously into 3 successive mice (0.3 mg., 470 HU per mouse), and they died in 8, 9 and 70 minutes, respectively. The second part of the hemolysin solution was treated with an excess of cholesterol suspension (0.05 mg. sterol per 0.3 mg. dose of hemolysin) to abolish all hemolytic activity quickly. As soon as possible after mixing (6, 12 and 15 min.), each of 3 mice was injected with the above dose; and another lot of 3 mice were injected with the mixture after it had been incubated for 30 minutes at 30°; all animals in both groups survived.

These results show that the hemolysin, when treated with cholesterol, loses its toxicity as well as its hemolytic activity. The same type of experiment was performed by Hewitt and Todd (11) upon extracts containing streptolysin O with similar results.

Immunization against the lethal effect. To determine if injection of the hemolysin would result in immunization, each of six mice was injected intraperitoneally with a total of 2 mg. of active concentrate

59–61 (2400 HU per mg.) over a period of 6 days. After a rest of 10 days, these animals were tested with an intravenous toxic dose of concentrate 60 IX. The amount injected (0.25 mg., 350 HU) was known from the toxicity curve to be well over the L_{100} dose. Three untreated mice received the same test dose as controls. The results were as follows:

Pre-treated mice:	D5′,	S,	S,	S,	S,	S
Controls:	D1.5′,	D3′,	D5′			

It seems therefore that mice are capable of developing an antitoxic immunity to the hemolysin. The one fatality in the immunized group of six may have been due to an individual deficiency in immunological response, or it may be indicative of an irregular and perhaps quantitatively low response. The surviving immunized mice appeared rather sick for an hour or two after receiving the test dose of hemolysin.

Dermotoxicity

A. Skin reaction with active hemolysin. The intracutaneous injection of the active hemolysin in rabbits, guinea pigs and mice brought forth local dermotoxic reactions characterized by necrosis. The course of the process is illustrated in the following experiment.

Into each of 3 guinea pigs[3] there was injected intracutaneously 0.1 ml. of a buffered solution containing 1 mg. (2400 HU) of preparation 59–61. The following sequence of changes occurred at each site of injection.[4]

5 to 10 minutes: purple zone with red halo;

1 hour: solid, intense purple area, 10 to 15 mm. in diameter;

[3] These animals also received at the same time, and in adequately spaced skin areas, injections of sterile phosphate buffer as well as the injections described in the following sections B and C.

[4] The development of the skin necrosis in the CFl mice after injection of hemolysin concentrate was found to be qualitatively similar to that observed in the guinea pig, but the process was much slower. The purplish discoloration appeared in 2 days, the central necrosis with red halo appeared during the third day, and healing to scar formation took up to 15 days. The cause of this difference in the rate of development of the skin lesion seems to be a species effect related, perhaps, to the high resistance of the mouse red cells to the hemolysin (see above).

2 hours: yellow necrotic center (3 to 5 mm. diam.) within the purple area;
6 hours: necrotic center spread over whole of previously purple area;
1 to 2 days: scab formation begun and gradually becoming well-developed; 9 to 10 days: complete healing.

In several experiments, an attempt was made to titrate the dermotoxic effect in guinea pigs by the intradermal injection of decimal dilutions of the concentrates and measuring the areas of necrosis that developed in 48 hours. Three preparations of widely differing hemolytic potencies were used, viz.: 63A2, 6000 HU per mg.; 59–61, 2400 HU per mg.; and 64B, 700 HU per mg. These experiments need not be cited in detail because the results were difficult to quantitate. Nevertheless the outcome rather consistently indicated the following general tendencies. (a) For any one preparation, there was a rough parallel between the amount of hemolysin injected and the area of the resulting skin necrosis. (b) As between samples of high and low hemolytic potency, the area of necrosis that each produced was roughly proportional to the number of hemolytic units injected. (c) The minimal dose required for the production of grossly detectable dermonecrosis in the guinea pig varied from 15 to 50 HU.

B. Skin reaction with heat-inactivated hemolysin. Preparation 59–61 in buffer pH 7.6 was heated for 10 minutes at 60°, which completely abolished its hemolytic activity. This material (1 mg. in 0.1 ml.) was injected intradermally into each of 3 guinea pigs. No skin necrosis occurred at the sites of these injections.

C. Skin reaction with cholesterol-inactivated hemolysin. Part of the solution of preparation 59–61 was treated with an aqueous suspension of cholesterol. Enough of the latter was added and allowed to act for 10 minutes at room temperature to reduce the hemolytic titer in the 0.1 ml. dose from 2400 to 16 HU. The intradermal injection of this hemolysin-cholesterol mixture into the above 3 guinea pigs produced no necrosis.

D. Skin reaction with reversibly oxidized hemolysin. Preparation 61-2CCC, 90% reversibly oxidized, was dissolved in buffer so that the 0.1 ml. dose contained 160 active (plus 1440 potential) HU. This was injected intradermally into 2 guinea pigs and produced the characteristic skin reaction, except that it was somewhat delayed. As reported

above, the fully active hemolysin produced the first sign of skin necrosis in the guinea pig in about 2 hours; but with the reversibly oxidized preparation, it took about an hour or two longer before the first signs of necrosis appeared.

E. Skin reaction with irreversibly oxidized hemolysin. Part of the solution of preparation 59–61 was oxidized with iodine solution to abolish the hemolytic activity irreversibly, and part was left untreated. Each of these solutions, in the usual dose of 1 mg. in 0.1 ml., was

TABLE II

Specific neutralization of skin necrosis in rabbit

Concentrate 63A2, 3.3 mg. per ml. in phosphate buffer 7.6.
Rabbit serum 25-4B, antihemolysin titer 0.00005 ml. = 1 HU of 63A2.
Control normal rabbit serum, antihemolysin titer: 0.05 ml. = 1 HU.
Intradermal dose 0.1 ml. Observation at 27 hrs.

MIXTURE NO.	HEMOLYSIN		ANTISERUM		NORMAL SERUM ml.	SKIN REACTION*
	mg.	HU	ml.	Units		
1	0.033	200			0.09	Purple; necrosis?
2	0.033	200	0.09	1800		Pink; no necrosis
3	0.165	1000			0.05	Purple; necrosis, 5 mm.
4	0.165	1000	0.05	1000		Erythema; no necrosis
5	0.300	1800			0.01	Purple; necrosis, 10 mm.
6	0.300	1800	0.01	200		Purple; necrosis, 2 mm.
7	0.300	heated			0.01	Pink; no necrosis
8	0.300	1800				Purple; necrosis, 10 mm.
9			0.09	1800		Pink; no necrosis
10					0.09	Pink; no necrosis

* Area of necrotic center, if present, indicated by diameter in mm.

injected intradermally into the above two guinea pigs. No necrosis occurred with the irreversibly oxidized hemolysin; but typical necrosis occurred with the hemolytically active substance.

F. Specific neutralization of dermotoxic activity with antiserum. Specific neutralization of the dermonecrotic activity was tested in experiments upon the albino rabbit. Different proportions of concentrate 63A2 and antihemolysin serum (rabbit) were mixed, incubated at room temperature for 30 minutes and tested along with the necessary controls. The results, given in Table II, indicate that specific neutralization of the dermotoxic activity had occurred. Mixture 1 produced

a marked purple discoloration but questionable necrosis, whereas mixture 2 containing the same amount of hemolysin plus a large excess of antiserum left no effect except a transient reddening of the skin. Mixtures 3 and 4 gave evidence of complete neutralization; and partial neutralization was indicated by mixtures 5 and 6.

F. Skin reaction with hemolysin after absorption with erythrocytes. The evidence adduced thus far indicates that the hemolytic and dermotoxic activities of the hemolysin preparations are closely associated. This may mean that the two activities reside in one substance, or that they are due to two distinct substances very much alike, but differing in their affinities for certain cells. To test the latter possibility, attempts were made to absorb the hemolytic activity of four different concentrates with erythrocytes so as to leave the dermotoxic activity alone or, at least, predominant.

Rabbit erythrocytes were freshly washed with M/15 phosphate buffer, pH 7.6, and lightly spun out. The hemolysin preparations were dissolved in the same buffer in a concentration of 10 mg. per ml. All operations in the absorptions were carried out at 0° to 2°C with chilled glassware. Each hemolysin solution (0.4 to 0.9 ml.) was added to 1 ml. of lightly packed cells, stirred for 1 to 2 minutes, spun out for 4 to 5 minutes and the supernate removed for tests of residual hemolysin and dermotoxic activity. None of the absorptions was either complete or without accompaniment of a slight degree of hemolysis which was generally proportional to the activity of the preparation used.

Four different hemolysin concentrates were thus treated and injected intradermally along with the corresponding unabsorbed solutions into guinea pigs and the results are recorded in Table III.

It can be seen that the erythrocytes removed from 92 to 97% of the hemolytic activity of each of the first three preparations listed in Table III and that dermotoxic activity was not manifested after doses of 24 and 32 HU, but did appear after a dose of 96 HU. Recalling the considerable disparity in accuracy of the tests for hemolytic and for dermotoxic activity and the fact that about up to 50 HU were required for the production of detectable dermonecrosis in the guinea pig, the results are interpreted as indicating that the dermotoxic activity of the concentrates disappeared as the specific absorption of the hemolytic activity was more nearly complete.

Incidentally, it may be noted in Table III that treatment of active hemolysin with red cells, even at 0° to 2°C, resulted in an appreciable percentage increase of reversibly oxidized hemolysin in the recoverable material. Of interest also is the behavior of preparation 61-2CCC which was 90% reversibly oxidized at the start. As was to be expected, it remained generally unabsorbed by the erythrocytes (3); but there was a diminution of 50 active HU and 60 inactive HU, as if progressive oxidative change, or else non-specific absorption on the erythrocytes, had taken place.

TABLE III

Hemolytic and dermotoxic activities of concentrates before and after absorption with erythrocytes. (See text)

PREPARATION	INJECTED BEFORE ABSORPTION			INJECTED AFTER ABSORPTION		
	Total HU	Oxidized[1] (%)	Skin necrosis[2]	Total HU	Oxidized[1] (%)	Skin necrosis[2]
59-61	800	0	+	24	60	0
63A2	1200	0–30[3]	+	96	75	+
64B	450	20	+	32	60	0
61-2CCC	750	90	+	640	96	+

[1] Reactivable by reduction with cysteine or H_2S.

[2] At 48 hours.

[3] Partly oxidized during manipulation.

Immunization against experimental pneumococcal infection. A series of 100 CFl white mice received six daily intraperitoneal injections of increasing amounts of concentrate 59–61. The total amount injected was 2 mg. (4800 HU). Control animals were similarly injected with sterile buffer solution. Ten days after the last injection, groups of the animals were infected intraperitoneally with serial dilutions of broth cultures (6 to 8 hours old) of virulent Type I or Type II pneumococci. After injection of Type I pneumococci, 5 out of 10 immunized mice survived 100 fatal doses, and 9 out of 10 survived 10 fatal doses. With Type II pneumococci, 5 out of 10 immunized mice survived 1000 fatal doses, and 8 out of 10 survived 100 fatal doses. Such results indicate that active immunization of the mice with the hemolysin concentrate produced a definite but low grade, non-type-specific protection against the infections.

The protocols need not be presented in detail because there is no certainty that the observed immunizing effect was due to the hemolysin in the concentrate, or that such immunization plays a significant role in protection against the onset of pneumococcal infection. However, the observation may be of interest because Dubos (5) has found a similar response of mice to immunization with a nucleohistone-containing soluble antigen extracted from pneumococci and because immunization with dead rough pneumococci can produce a similar heterologous response (19).

Antihemolysin

The observations of Cole (4), and especially those of Neill and his associates (14, 15), show that the hemolysin in pneumococcal extracts is capable of eliciting a specific antibody which neutralizes the hemolytic activity of the substance. The following experiments upon the concentrates confirm and extend the immunological evidence on the position of the hemolysin in the antigenic complex of the pneumococcus.

Antigen. The material injected was concentrate 60 IX containing 1400 HU per mg. This specimen was one of the few that was "purified" by reprecipitation, wherein the starting material (of about 2000 HU per mg.) was dissolved in water, clarified by centrifugation, and reprecipitated with acetic acid at pH 4.5. There occurred some loss in specific hemolytic activity due to irreversible deterioration; nevertheless, this material was considered to be less contaminated than the simple concentrates, and therefore more suitable for the production of specific antiserum.

Immunization. The concentrate was injected intravenously into four rabbits in three series as follows: 6 daily doses of 0.5 mg., 5 daily doses of 1 mg., and 5 daily doses of 3 mg., respectively, with 7-day rest periods between each series. A fourth series of injections (successive daily doses of 1, 2, 3, 4, 5, and 5 mg.) was made 3 months later. Thus, each animal received a total of 43 mg. of the concentrate. In all cases the amount for injection was contained in 0.5 or 1.0 ml. of sterile phosphate buffer, pH 7.6. Samples of the respective sera were obtained before immunization and seven days after each series.

Antihemolysin titration. The specific potency of the sera was determined as follows. Serial dilutions of each serum were mixed and

incubated for 10 to 45 minutes with a known number of hemolytic units of hemolysin to allow combination between antigen and antibody. After this, a fixed amount of red cells was added to determine the amount, if any, of unneutralized hemolysin. In this way, the minimum volume of serum necessary to neutralize 1 HU of active hemolysin was obtained. A test of the method using appropriate dilutions of antiserum 25-4B against 1.9, 3.8, 7.6 and 15.2 HU of active concentrate 59A yielded the values 0.00007, 0.00022, 0.00020, and 0.00010 ml., respectively, of the antiserum as the neutralizing equivalent of 1 HU of hemolysin.

The antihemolytic titer of the normal rabbit sera was small, varying from 0.01 to over 0.1 ml. After the first series of immunizing injections, the titers reached 0.002 to 0.006 ml.; after the second series, they varied from 0.0007 to 0.0010 ml.; and after the third series, they reached 0.00005 to 0.0003 ml. The fourth series produced slight but hardly significant improvements in the antihemolytic titers of the four immune sera.

An indication of the antihemolytic specificity of these immune sera may be gained from the following experiment with antiserum 21-4B against three unpurified concentrates of widely differing hemolytic potencies, namely, 64B (700 HU per mg.), 59A (1500 HU per mg.), and 63A1 (6000 HU per mg.). The serum yielded antihemolytic titers of 0.00011, 0.00010, and 0.00014 ml. per hemolytic unit of the respective samples. Thus, these preparations were neutralized by the antiserum in proportion to their content of hemolysin.

Precipitin Reactions

The hemolysin used in these experiments was derived from a rough strain of Type II pneumococcus and its content of type-specific capsular polysaccharide was below the limit detectable by the precipitin and agglutination tests reported below. In addition, precipitin tests were performed to detect the presence of C carbohydrate in the concentrates, and precipitin absorptions were carried out to discover possible relations between hemolysin antibody and C antibody.

The precipitin titers of the antihemolysin sera (rabbit) were determined by layering, and later mixing, 0.2 ml. of the undiluted serum with 0.2 ml. of serial dilutions of the antigen. The layered solu-

tions were incubated for 2 hours at 37°C, and then shaken and refrigerated. The final readings were made after 24 to 72 hours. Positive and negative controls were included in each series of tests.

The antihemolysin sera yielded precipitates with active concentrates 59A and 63A2 down to dilutions of 1:100,000 to 1:800,000 (mean, 1:400,000). A much lower precipitin titer, 1:20,000, was given by the crude preparation, 64B, which was judged from its low hemolytic potency (700 HU per mg.) to contain much extraneous matter. With the 90 per cent reversibly oxidized concentrate 61-2CCC, precipitation occurred down to the same limit of dilution, namely 1:100,000, and the precipitin titer did not change when cysteine was added to the mixture. In normal control sera, these antigens at dilutions of 1:200 to 1:100,000 produced no detectable precipitate. Concentrate 63A2, after being heated at 55°C for 10 minutes to render it hemolytically inactive, retained part of its ability to form precipitates in the antihemolysin sera,—the limiting dilution was 1:100,000 as compared with 1:800,000 for the unheated preparation.

With the specific capsular polysaccharides SSS I and SSS II in dilutions down to 1:6,000,000, the antisera produced slight precipitation only at 1:200 to 1:2,000 dilutions of antigen, like the normal control sera. A Type II antipneumococcus serum (rabbit), that precipitated SSS II in 1:4,000,000 dilution and had a low (0.01 ml.) antihemolysin titer, gave no precipitate with the concentrates, except at the limit of the non-specific zone, 1:2,000 to 1:10,000. These negative precipitin tests were confirmed by the agglutination tests kindly performed for us by Dr. J. Howard Brown. Two of these antisera were examined for agglutinins against Types I and II pneumococci and were found negative in dilutions from 1:20 to 1:1280.[5]

Experiments with C carbohydrate. We are indebted to Dr. Lloyd D. Felton for drawing our attention to the fact that the method of preparing the concentrates left open the possibility that the hemolysin might be related to or contaminated with the species-specific carbohydrate, the "C substance", first described by Tillett and Francis (20) and further characterized by Heidelberger and Kendall (9), and by Goebel and associates (6, 7). Information was sought through the respective

[5] These antisera were found to agglutinate Type III pneumococci in dilutions of 1:20 to 1:160.

antigens, the antihemolysin sera and selected anti-C horse sera of high titer. The antibody content of the various antisera was undetermined. Typical results of the precipitin tests are shown in Table IV, which also gives for comparison the antihemolytic titers of the respective sera.

Table IV shows that antihemolysin serum 25-4B, with high specific antihemolytic and precipitin titers, yielded little or no specific precipitates with the C carbohydrate. On the other hand, the anti-pneumococcus sera selected for their high anti-C titers gave moderate to high precipitin titers with the hemolysin.

TABLE IV

Antihemolytic and precipitin titers compared

SERUM	ANTIHEMOLYTIC TITER	PRECIPITIN TITERS WITH:		
		Hemolysin 63A2	C_I‖	C_{II}‖
	ml.			
Normal rabbit	0.1–0.01	1–200§	< 20,000	20,000
25-4B rabbit*	0.0001–.0002	800,000	10,000	< 20,000
H-884 (Horse)†	0.01	100,000	2,000,000	2,000,000
FH_3 (Horse)‡	0.002	200,000	2,000,000	
$FH_{5,7}$ (Horse)‡	0.0003	800,000	6,000,000	

* Antihemolysin serum produced with concentrate 60 IX.

† Type I antipneumococcus serum kindly supplied by Dr. Michael Heidelberger.

‡ Type II and mixed Types V and VII antipneumococcus sera kindly supplied by Dr. Lloyd D. Felton.

§ The numbers are reciprocals of the highest dilutions of antigens producing detectable precipitation.

‖ C carbohydrates prepared from Types I and II pneumococci, respectively, by Dr. Michael Heidelberger. The preparations contained small amounts of homologous capsular polysaccharide.

It was also found that the high specific precipitin titer of antipneumococcus horse serum $FH_{5,7}$ with hemolysin was slightly diminished after the serum was absorbed with the C carbohydrate. Likewise, absorption of antihemolysin rabbit serum 25-4B with excess of C carbohydrate caused a similar slight decrease in its precipitin titer with concentrate 63A2. This diminution in precipitins was apparently paralleled, in a preliminary test, by a corresponding decrease in the inhibiting effect of the treated serum upon the action of the hemolysin on red cells. However, this detail requires further study.

Cross absorptions were made of the precipitins for the hemolysin

and for the C carbohydrate in the three selected antipneumococcus horse sera previously mentioned. Each antigen was added in excess to a sample of the serum, the mixture incubated and then spun until clear, and the supernatant was finally titred with the other antigen. Absorption with the hemolysin concentrate left each antiserum practically free of specific precipitins for both the C carbohydrate and the hemolysin. On the other hand, absorption with the C carbohydrate removed all of the specific C precipitins but left a considerable proportion of the specific hemolysin precipitins unabsorbed. A sample of the experimental results with serum $FH_{5,7}$ is shown in Table V.

TABLE V

Cross-absorption of precipitins for hemolysin and C carbohydrate

PRECIPITATING ANTIGEN	SERUM $FH_{5,7}$* (DILUTED 1:6)		
	Unabsorbed	Absorbed with C_1	Absorbed with 59A
Hemolysin 59A	+200,000† ±400,000	+100,000 ±200,000	±500 −1,000
C carbohydrate (C_1)*	+2,000,000‡	+10,000 −20,000	+20,000 −100,000

* See Table IV.

† The numbers are reciprocals of the highest dilutions of antigens producing: + = definite precipitation; ± = barely perceptible precipitation; − = no precipitation.

‡ Highest dilution tested.

DISCUSSION

The foregoing observations throw additional light on the biological properties of the hemolysin. This constituent of the pneumococcus has been shown to possess the properties of a toxin; and the evidence strongly suggests that its hemolytic (*in vitro*), lethal and dermotoxic activities reside in one and the same protein component of the concentrates studied.

A lethal intravenous dose of the hemolysin was usually rapidly fatal. In this respect the substance resembles streptolysin O and the toxins of staphylococcus, *Clostridium welchii* and *C. septicum* which do not exhibit a latent period such as is observed in the case of diphtheria and tetanus toxins (cf. (1)). Apart from the observed pulmonary congestion to edema, the site of action of the hemolysin was undetermined. The observed symptoms are, however, consistent with a diagnosis of circu-

latory failure as the cause of death. This inference finds support in the recent observations of Bernheimer and Cantoni (1) that concentrates of streptococcal and pneumococcal hemolysins produce cardiospasm in the isolated frog's heart.

In no case were any purpuric manifestations observed in the many mice that received intravenous injections of the concentrates. The absence of this symptom indicates the absence from the preparations studied of significant amounts of the heat-stable, purpura-producing substance described by Julianelle and Reimann (12) as an autolytic product of pneumococci. Hemoglobinuria or other gross evidence of intravascular hemolysis was not observed in any of the mice killed by the hemolysin concentrates; surviving mice were not examined. The non-appearance of intravascular hemolysis either grossly or in the tissue sections, may have been due in part, at least, to a species or strain characteristic of the mice employed, in that the washed erythrocytes of the CF1 mouse were abnormally resistant to the hemolysin. However, further study is required to elucidate the interaction of hemolysin and the red cell *in vivo*. In the case of mice killed by streptolysin O, Todd (21) observed that intravascular hemolysis was not conspicuous *post mortem*, although hemoglobinuria in occasional surviving animals indicated that hemolysis *in vivo* did occur sometimes.

The LD_{50} in mice weighing 25 to 30 gm. was found to be 0.12 to 0.13 mg. with concentrates of 1400 to 1500 HU per mg., and approximately 0.04 mg. with a concentrate of 6000 HU per mg. hemolytic potency. This indication that the acute lethal toxicity was associated with the hemolytic activity is supported by the additional evidence that in all cases tested a treatment which abolished the hemolytic activity of a concentrate also abolished the lethal toxicity (see Table VI). Perhaps the most impressive experiment in this connection is the one showing that the reversibily oxidized hemolysin was non-toxic, whereas the same dose when reactivated with cysteine was toxic. Incidentally, since both the reduced and the reversibly oxidized hemolysins are apparently able to elicit the same specific antihemolysin (14), it would appear that the two substances stand toward each other in the relation of toxin to toxoid. Both react with specific antisera, although the reversibly oxidized form apparently binds less antibody than does the reduced form (unpublished experiments).

The fact that the cholesterol-treated concentrate is non-toxic suggests that the combination of hemolysin and cholesterol is not readily broken apart in the body. This inference finds support in the observation (2) that exhaustive extraction of cholesterol-treated hemolysin with benzol, under conditions which left the untreated hemolysin unchanged in hemolytic activity, was not able to restore more than a small fraction of the original activity. In addition, the cholesterol-treated concentrate appears to be antigenically weaker, because three rabbits immunized with it yielded sera of relatively weak antihemolytic titers (0.001, 0.003, and 0.007 ml. per HU). There is another aspect of the subject that poses an interesting problem, namely, the failure of

TABLE VI

Summarization of evidence for association of lethal toxicity and dermotoxicity with hemolytic activity of hemolysin concentrates

TREATMENT OF HEMOLYSIN	LETHAL TOXICITY	DERMO-TOXICITY	HEMOLYTIC ACTIVITY (IN VITRO)
Irreversibly oxidized	not tested	0	0
Reversibly oxidized (90%)	0	+ (delayed)	±
Native (reduced)	+	+	+
Native heated	0	0	0
Native + cholesterol	0	0	0
Native absorbed with rabbit r.b.c.	not tested	±	±
Native + specific antiserum	not tested	0	0

0 means no activity; + means full activity; ± indicates slight or modified activity as described in the text.

the cholesterol normally present in the mouse blood to detoxify the intravenously injected hemolysin, especially since even 0.1 ml. of the serum should contain enough free cholesterol to detoxify several lethal doses. This failure to detoxify may possibly be due merely to the relative rapidity of the circulation as compared with the rate of combination of the hemolysin with the blood cholesterol.

The dermotoxic activity of the hemolysin was found also to be associated with the hemolytic activity in all but one of the tests that were performed. This association is summarized in Table VI. The one apparent exception is the behavior of the reversibly oxidized hemolysin. This was a preparation only 90% oxidized, and it therefore

exhibited a residual hemolytic activity. It had no lethal toxicity in the dose employed but showed a somewhat delayed yet full dermotoxic activity which can be accounted for physiologically. The intradermal tissue represents a region of appreciable metabolic activity associated with the production of reducing metabolites which in due time, depending on the amount of reversibly oxidized hemolysin injected, should be able to reduce it to the active, toxic state. It has been shown (13) that the oxidized hemolysin is reducible to its active hemolytic state when incubated with actively growing *Escherichia coli* and a species of anaerobic bacillus.

The experiments designed to test the separability of the hemolytic and dermotoxic properties of several of the concentrates showed that the dermotoxicity disappeared as the specific absorption of the hemolytic activity by erythrocytes was more nearly complete. The association of these properties was such as to indicate that they belonged to one substance. In this respect, the hemolysin concentrate seems to differ from the anaerobic autolysate described by Parker (16) as possessing hemolytic and lung-toxic activities which could be separated by means of rabbit erythrocytes from the dermotoxic activity.

The immunological observations recorded in this report show that the hemolysin concentrate, in addition to its ability to elicit in rabbits the production of specific antibody that neutralizes the hemolytic activity, can also actively immunize mice against the lethal toxic effect: a total of ten out of twelve immunized animals were found to withstand the subsequent injection of over twice the LD_{50}. Adequate tests of toxin-antitoxin neutralization were not carried out. In respect to the dermotoxic activity, evidence indicating specific neutralization by antihemolysin serum was obtained in our experiments.

The method of separating the hemolysin was such as to permit the extraction of at least some of the C carbohydrate, and the precipitin reactions indicate that it was present in the several preparations tested. This was shown by the tests on the selected horse sera that had high anti-C and antihemolysin titers. Absorption of these antisera with C carbohydrate removed the specific C precipitins but left most of the hemolysin precipitins unabsorbed; whereas absorption with hemolysin concentrate removed the precipitins for both the C carbohydrate and the hemolysin. Further study may be required to decide whether the

uniform but small reduction observed in the antihemolysin and precipitin titers of the sera after absorption with C carbohydrate was an artifact or an indication of an antigenic relationship between hemolysin and C carbohydrate. The observation that antihemolysin rabbit serum gave no specific precipitate with C carbohydrate can be taken to indicate that the hemolysin concentrate contained no effective amounts of this carbohydrate in antigenic form. However, it may be necessary to rule out the presence of the C-lipid complex (heterophile antigen) from the hemolysin concentrate, because rabbits immunized with the lipid complex produce antibodies which are weak in C precipitins (6). There is no information as yet as to the quantities of C carbohydrate in the concentrates studied, except that they had roughly 10% of the precipitating activity of available C preparations in anti-C sera.

Tests have shown that the C carbohydrate itself does not hemolyze washed rabbit erythrocytes; and there is no indication that it participates in the lethal and dermal toxicities, or the *in vitro* hemolytic activity of the concentrate. These three known biological properties of the hemolysin concentrate seem to be associated with the protein contained therein. Furthermore, the fact that in every case tested (see Table VI), a treatment that affected one of these properties affected the others in essentially the same manner suggests strongly that they represent three different manifestations of one and the same protein.

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SUMMARY

Concentrates containing hemolytically active (reduced) pneumococcal hemloysin were shown to possess lethal and dermotoxic activities. The reversibly oxidized hemolysin was non-hemolytic and non-lethal, and produced somewhat delayed dermonecrosis. Brief exposure of the concentrates to heat at 60°C, or to cholesterol, abolished the hemolytic, lethal and dermal activities. Absorption with rabbit erythrocytes appeared to remove the hemolytic and dermotoxic activities at approximately the same relative rates. Mice immunized with the concentrate were generally capable of withstanding a subsequent

injection of at least twice the LD_{50}. The dermotoxic activity was found to be neutralized by specific antihemolysin serum.

The presence in the hemolysin concentrates of C carbohydrate, presumably in non-antigenic form for the rabbit, was indicated by the results of precipitin and precipitin-absorption tests.

The evidence indicates that the hemolysin has the properties of a toxin, and strongly suggests that its hemolytic (*in vitro*), lethal and dermotoxic activities reside in one and the same protein component of the concentrates.

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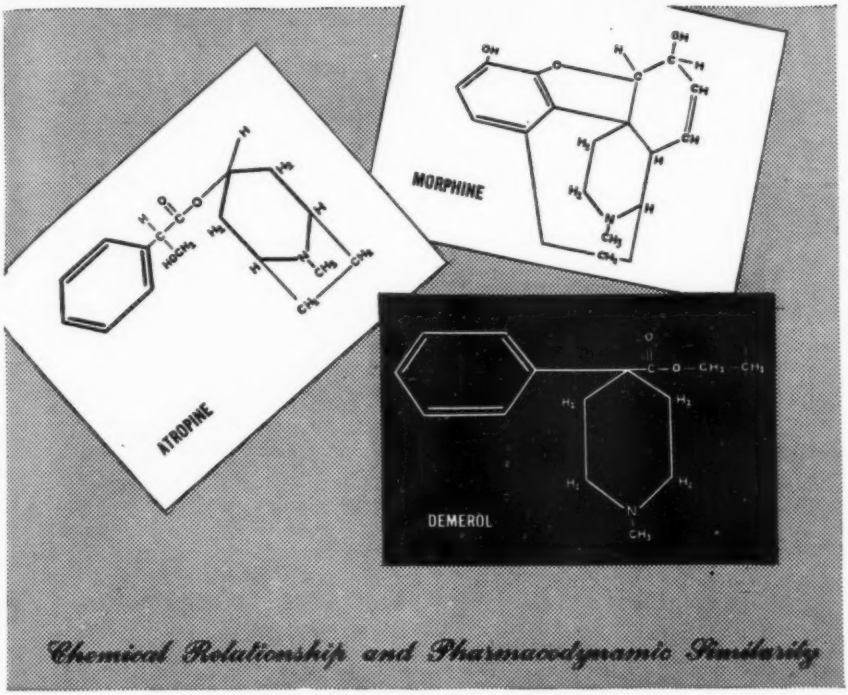

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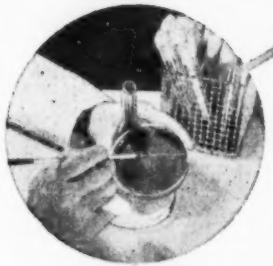

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